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2,3,7,8-Tetrachlorodibenzo-p-dioxin activates the aryl hydrocarbon receptor and alters sex steroid hormone secretion without affecting growth of mouse antral follicles in vitro

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Abstract

The persistent environmental contaminant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is an ovarian toxicant. These studies were designed to characterize the actions of TCDD on steroidogenesis and growth of intact mouse antral follicles in vitro. Specifically, these studies tested the hypothesis that TCDD exposure leads to decreased sex hormone production/secretion by antral follicles as well as decreased growth of antral follicles in vitro. Since TCDD acts through binding to the aryl hydrocarbon receptor (AHR), and the AHR has been identified as an important factor in ovarian function, we also conducted experiments to confirm the presence and activation of the AHR in our tissue culture system. To do so, we exposed mouse antral follicles for 96 hours to a series of TCDD doses previously shown to have effects on ovarian tissues and cells in culture, which also encompass environmentally relevant and pharmacological exposures (0.1-100nM), to determine a dose response for TCDD in our culture system for growth, hormone production, expression of the *Ahr* and *Cyp1b1*. The results indicate that TCDD decreases progesterone, androstenedione, testosterone, and estradiol levels in a non-monotonic dose response manner without altering growth of antral follicles. The addition of pregnenolone substrate (10μM) restores hormone levels to control levels. Additionally, *Cyp1b1* levels were increased by 3-4 fold regardless of the dose of TCDD exposure, evidence of AHR activation. Overall, these data indicate that TCDD may act prior to pregnenolone formation and through AHR transcriptional control of *Cyp1b1*, leading to decreased hormone levels without affecting growth of antral follicles.

Keywords

TCDD; antral follicle; proliferation; ovary; steroidogenesis; aryl hydrocarbon receptor

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Conflict of Interest Statement

The authors have no conflicts to disclose.

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Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a persistent environmental contaminant inadvertently produced as a by-product of herbicide and pesticide manufacturing (Hites, 2011). TCDD is also released during the bleaching process at tree pulp and paper mills, and during burning of municipal solid waste (Frakes *et al.*, 1993; Tuppurainen *et al.*, 2003). TCDD is the most toxic member of a class of chemicals called dioxins. Dioxins, like TCDD, have a long environmental half-life, bioaccumulate in the food chain, and can be found in human fat tissue, blood serum, breast milk and ovarian follicular fluid (Birnbaum and DeVito, 1995; Humblet *et al.*, 2011; Schecter *et al.*, 2006; Tsutsumi *et al.*, 2011; Ulaszewska *et al.*, 2011).

Studies of human populations accidentally exposed to high levels of TCDD and controlled studies using various animal models exposed to TCDD have shown that TCDD is a potent endocrine disruptor. TCDD exposures have been linked to delayed puberty and early onset of menopause in women (Eskenazi *et al.*, 2005; Warner *et al.*, 2007). Similarly, TCDD exposures lead to early puberty, irregular estrous cycles, reduced or blocked ovulation, decreased circulating estradiol levels (E2), and early reproductive senescence in female rodents (Gray and Ostby, 1995; Li *et al.*, 1995; Chaffin *et al.*, 1996; Franczak *et al.*, 2006; Jablonska *et al.*, 2010; Myllymäki *et al.*, 2005; Shi *et al.*, 2007).

Though it has been shown that TCDD can affect the development and functioning of the rat hypothalamus and pituitary, there is convincing evidence that it has direct effects on the ovary as well (Cao *et al.*, 2011; Gao *et al.*, 2001; Mizuyachi *et al.*, 2002; Petroff *et al.*, 2001; Petroff *et al.*, 2003). Further, it has been demonstrated that TCDD can accumulate in ovarian follicles following controlled dosing experiments in animals and in culture (Baldridge and Hutz, 2007; Grochowalski *et al.*, 2001).

Antral follicles are the main functional unit of the ovary, housing the gametes and serving as the primary source of sex steroid hormones, such as 17 β -estradiol (E2). Follicles grow from the primordial to the antral stage in a process called folliculogenesis. In the process of folliculogenesis, follicles increase in size mainly due to proliferation of granulosa cells. It has been proposed that TCDD decreases circulating E2 levels in part by inhibiting the growth of follicles from the preantral to antral stage and by disrupting the synthesis and metabolism of sex steroid hormones (Dasmahapatra *et al.*, 2000; Gregoraszczuk *et al.*, 2001; Grochowalski *et al.*, 2001; Heimler *et al.*, 1998b; Heimler *et al.*, 1998a; Morán *et al.*, 2003a; Morán *et al.*, 2000; Morán *et al.*, 2003b; Myllymäki *et al.*, 2005; Pesonen *et al.*, 2006). In the ovary, E2 synthesis requires both the thecal and granulosa cell compartments. In the rodent, the thecal cells are responsible for producing progesterone (P4) and androstenedione (A4). A4 then diffuses to nearby granulosa cells where it is converted to testosterone (T) and E2. In antral follicles, the rate limiting steps in steroid hormone production are the mobilization of cholesterol from the cytoplasm to the inner mitochondrial membrane via steroidogenic acute regulatory protein (StAR) and the conversion of cholesterol to pregnenolone by the mitochondrial enzyme cytochrome P450, family 11, subfamily a, polypeptide 1 (P450scc) (Edson *et al.*, 2009). Pregnenolone is then converted to P4, and then by a multistep process to the androgens, and finally to E2.

To date, the majority of studies conducted with the purpose of understanding the mechanism underlying disrupted follicle growth and female sex steroid hormone production have been conducted using isolated granulosa or thecal cells in vitro. Additionally, the results reported vary widely and sometimes are conflicting depending on the cell line, culture system, animal model, dose of TCDD, length of exposure, and age of the animals being used. Thus, in this study, we set out to determine the effect of various doses of TCDD on intact adult mouse

antral follicles on growth and steroid hormone production/secretion. We specifically tested the hypothesis that TCDD exposure leads to decreased sex hormone production/secretion by antral follicles as well as decreased growth of antral follicles in vitro. Since TCDD is known to act through binding to the transcription factor, the aryl hydrocarbon receptor (AHR), and the AHR has been identified as an important factor in ovarian function (Hernández-Ochoa *et al.*, 2009; Rowlands and Gustafsson, 1997), we also conducted experiments to confirm the presence and activation of the AHR in our tissue culture system. Since our results indicate that TCDD inhibits steroidogenesis in antral follicles, we further investigated whether we could restore steroid production/secretion in TCDD exposed follicles by co-treating with pregnenolone, an early substrate in steroidogenesis.

Material and Methods

Chemicals

2,3,7,8-tetrachlorodibenzo-p-dioxin dissolved in dimethyl sulphoxide (DMSO) at 50 µg/mL (#ED-901-B) was purchased from Cambridge Isotope Laboratories, Inc., Andover, MA. Pregnenolone (P9129), DMSO (D2650), 100X insulin, transferrin, and selenium (ITS), penicillin and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO). Alpha minimal essential media (α-MEM) was purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, Lawrenceville, GA. Human recombinant follicle-stimulating hormone (hFSH) was obtained from Dr. A. F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA.

Animals

CD-1 mice were purchased from Charles River and maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the University of Illinois Animal Care and Use Committee. Upon arrival to the University of Illinois animal care facility, mice were allowed to acclimate for a minimum of 48 hours prior to tissue collection. All mice were housed under strict 12L:12D lighting and temperature was maintained at 22±1°C. Food and water were provided *ad libitum*.

In vitro mouse antral follicle culture

Female mice were sacrificed by CO₂ asphyxiation followed by cervical dislocation on postnatal day (PND) 33 and their ovaries removed. Small antral follicles were isolated mechanically from the ovaries of 2-4 mice for each experiment based on relative size (200-350 µm) and cleaned of interstitial tissue using ne #5 watchmaker forceps in unsupplemented α-MEM. Approximately 20-30 antral follicles were isolated from each mouse. Follicles were then placed randomly one per individual well in a 96-well culture plate with α-MEM prior to treatment. For each experiment, a minimum of eight follicles were plated per treatment group. Supplemented α-MEM was prepared with 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium), 100 U/ml penicillin, 100 µg/ml streptomycin, 5 IU/ml hFSH, and 5% FBS. To determine a dose response for TCDD in our culture system (0.1nM-100nM), 10 fold serial dilutions of TCDD were prepared from the original stock with vehicle DMSO (133 µM, 13.3 µM, 1.33 µM, and 0.133 µM). The individual dilutions or vehicle alone were added to the supplemented α-MEM at an equal volume (0.75 µL/mL media) to maintain the vehicle concentration at a constant of 0.075% for each treatment. For treatment, unsupplemented α-MEM was removed from each well and replaced with 150 µL of supplemented α-MEM containing vehicle or TCDD. Follicles were then incubated for 96 h at 37°C in 95% air and 5% CO₂.

Similarly, for the cultures with TCDD and pregnenolone co-treatment, each chemical was added to the supplemented α -MEM such that each treatment contained the same volume of chemicals in combination or alone. Specifically, pregnenolone was solubilized in DMSO at 26.7mM and some of this stock was diluted two fold to 13.3mM. Two stock solutions of TCDD were made: 2.66 μ M and 1.33 μ M. Pregnenolone was added to the media for a final concentration of 10 μ M and TCDD was added to the media for a final concentration of 1nM. Finally, follicles were incubated with vehicle, pregnenolone (10 μ M), TCDD (1nM), or pregnenolone (10 μ M) and TCDD (1nM) for 96 h at 37°C in 95% air and 5% CO₂. At least 3 individual experiments were performed for each treatment.

Follicle growth analysis

Antral follicles were cultured as described above for 96 h. Follicle growth was examined at 24 hour intervals by measuring follicle diameter on perpendicular axes with an inverted microscope equipped with a calibrated ocular micrometer. Small antral follicles were considered as those having diameters of 200-350 μ m (Smitz and Cortvrindt, 2002). At least three separate culture experiments were performed for each chemical treatment. Follicle diameter measurements were averaged among treatment groups and plotted as percent change in diameter from time zero to compare the effects of chemical treatments on growth over time.

Measurement of sex steroid hormones by ELISA

Media were collected after the 96 hours culture and frozen at -80°C until they were subjected to enzyme-linked immunosorbent assays (ELISAs) for P4, A4, T, and E2 levels. ELISA kits were purchased from DRG Diagnostics (USA) and procedures were followed using the manufacturer's protocols. The media were randomly selected from 8-23 wells and were assayed individually for each treatment group from 3-6 experiments. The intra-assay and inter-assay variabilities were less than 10%.

Quantitative real time PCR analysis of *Ahr* and *Cyp1b1* transcript levels

At the end of each of the 96 hour cultures, 8-16 follicles from each treatment group were pooled and immediately snap frozen in liquid nitrogen and stored at -80°C until RT-qPCR analysis. Total RNA was extracted using the RNeasy Micro Kit with DNase treatment to eliminate potential genomic DNA contamination according to the manufacturer's instructions (Qiagen, Valencia, CA). Concentration, purity, and quality of the RNA were determined using the NanoDrop ND-1000 UV-Vis spectrophotometer at 260 nm and 280 nm (Nanodrop Technologies, Wilmington, DE). Total RNA (250ng) was reverse transcribed using an iScript cDNA synthesis kit according to manufacturer's instructions (Bio-Rad, Hercules, CA). Negative controls included omission of reverse transcriptase and omission of template. Each of the final cDNA samples were then diluted 1:4 for real time qPCR analysis.

All qPCR reactions were performed in triplicate using the CFX96 Real-time System C1000 Thermal Cycler (Bio-Rad). Each qPCR reaction contained 5 μ L of 2X SsoFast™ Evagreen® supermix, 0.6 μ L of gene specific primers, 2.4 μ L of nuclease free water, and 2 μ L of diluted cDNA template. The final concentration for each gene specific primer in the qPCR reactions was 0.3pmoles/ μ L. A BLASTN search was performed in GenBank to ensure that all primers were unique to the gene of interest. To avoid amplification from genomic DNA contamination, all primer sets spanned a large exon-intron-exon junction. Primer sequences were as follows: cytochrome P450, family 1, subfamily b, polypeptide 1 (*Cyp1b1*) Forward 5'-GCG ACG ATT CCT CCG GGC TG- 3', *Cyp1b1* Reverse 5' -TGC ACG CGG GCC TGA ACA TC- 3', aryl hydrocarbon receptor (*Ahr*) Forward 5'-TTC TTA GGC TCA GCG TCA GCT A- 3', *Ahr* Reverse 5' -GCA AAT CCT GCC AGT CTC TGA T -3', beta actin (*Actb*) Forward 5' -GGG CAC AGT GTG GGT GAC -3', *Actb* Reverse 5' -CTG GCA

CCA CAC CTT CTA C -3'. Initial denaturation of the cDNA and enzyme activation occurred at 95°C for 1 min, followed by 40 cycles of (10 seconds at 95°C, 10 seconds at 60°C, and a fluorescent absorbance reading), and one final annealing/elongation step for 5 minutes at 72°C. A heat dissociation curve (from 65°C-95°C with a fluorescent absorbance reading after each 0.5°C increment) was performed at the end of every run to assure specificity of each primer pair for the chosen transcript of interest.

For relative quantification of transcript levels, standard curves were generated using a six step serial dilution of cDNA generated from pooled RNA samples for each primer set being used (*Actb*, *Ahr*, and *Cyp11b1*). These standard curves were used to calculate the amplification efficiencies for each primer pair. Relative transcript amount was then calculated by a mathematical model developed by Pfaffl (Pfaffl, 2001). Briefly, the method involves calculating the relative expression ratio of the target gene based on the amplification efficiency of each amplicon and the ΔC_t of the treated samples versus the vehicle control. These ratios were then compared to the expression of the reference gene *Actb*. *Actb* was verified as a good internal control because its levels were unchanged with treatment (data not shown). The data were reported as mean transcript expression ratios relative to *Actb* from 3-4 separate follicle culture experiments.

Statistical Analysis

Least squares one-way analysis of variance (ANOVA) was used to analyze the data followed by the Tukey's *post hoc* test. If the data did not pass the assumption of homogeneity of variance, the non-parametric Kruskal-Wallis ANOVA was performed followed by the Mann-Whitney test. Statistical significance was assigned at $p = 0.05$ for all comparisons. All data were analyzed using SPSS 11.0 statistical software (SPSS Inc., Chicago, IL).

Results

Effect of TCDD exposure on antral follicle growth

Antral follicle growth is mainly characterized by proliferation of granulosa cells and accumulation of follicular fluid in the antral space leading to an increase in follicle volume over time (Hirshfield, 1991). To determine whether TCDD has an effect on follicle growth in vitro, follicle diameter was measured on a perpendicular axis every 24 hours for a period of 96 hours. TCDD had no effect on follicle growth at any of the four doses (0.1, 1, 10, and 100nM) when compared to vehicle alone (Fig 1). Additionally, follicles exposed to TCDD or vehicle had a similar gross morphology at the beginning and at the end of the 96 hour cultures. All follicles consisted of a single oocyte, an antrum, multiple layers of healthy granulosa cells, and a thecal layer surrounding the oocyte (Fig 2).

Effect of TCDD exposure on antral follicle steroid secretion

Antral follicles are the primary source of female sex steroid hormone production (Edson *et al.*, 2009). To determine whether TCDD has an effect on antral follicle steroid hormone production, follicles were treated with TCDD and the media were collected at the end of the 96 hour culture and assayed for levels of P4, A4, T, and E2. TCDD exposure for 96 hours resulted in U-shaped dose response curves for the amount of P4, A4, T, and E2 secreted into the media by the antral follicles in vitro when compared to vehicle alone (Fig 3). Specifically, the level of P4 was significantly decreased only at the 1nM dose of TCDD and not at the lowest (0.1nM) and two highest (10 and 100nM) doses of TCDD (Fig 3 a). The levels of androgens (A4 and T) were significantly decreased at the two lowest doses of TCDD (0.1 and 1nM), but not at the two highest doses (10 and 100nM) of TCDD (Fig 3 b and c). E2 levels were decreased after exposure to 0.1, 1, and 10nM, but not after exposure

to the highest dose of TCDD (100nM) (Fig 3 d). Interestingly, only the 1nM dose of TCDD significantly decreased the level of all four hormones simultaneously.

Effect of co-treatment of TCDD and pregnenolone on antral follicle growth and steroid secretion

To determine whether the decrease in hormone levels observed in the follicle culture media after 96 hours TCDD exposure was due to impaired StAR or P450scc function, pregnenolone was supplemented in the media with or without 1nM TCDD. The 1nM dose was chosen because this dose impairs the production or secretion of all four of the steroid hormones of interest (Fig 3). Antral follicle growth was monitored every 24 hours and the media were collected at the end of the 96 hour period for hormone assays. As demonstrated in the dose response experiments (Fig 3), exposure to 1nM TCDD for 96 hours again resulted in decreased production/ secretion of P4, A4, T, and E2 by the antral follicles in culture when compared to vehicle alone (Fig 4 a-d). Pregnenolone co-treatment restored hormone levels to control levels, suggesting a block in the steroidogenic pathway prior to pregnenolone formation, possibly via a StAR or P450scc impairment (Fig 4 a-d). Although pregnenolone co-treatment restored sex steroid hormone levels in the media, it did not alter antral follicle growth (Fig 5).

Effect of TCDD exposure on the expression of Ahr and Cyp1b1 in antral follicles

AHR activation by TCDD has been shown to increase metabolism of E2 by inducing the cytochrome P450 monooxygenases, such as CYP1B1 (Hayes *et al.*, 1996; Spink *et al.*, 2003). Thus, follicles were subjected to real time qPCR for levels of *Ahr* and *Cyp1b1* mRNA. TCDD exposure resulted in a three to four-fold induction of *Cyp1b1* expression in antral follicles regardless of the dose after 96 hours of culture, evidence of AHR pathway activation (Fig 6 a). Additionally, TCDD did not affect the expression level of *Ahr* mRNA in cultured antral follicles regardless of the dose (Fig 6 b).

Discussion

To our knowledge, studies have not reported the direct effects of TCDD on mouse antral follicles in vitro. In fact, only one published study has reported on direct effects of TCDD on steroidogenesis and growth in follicles isolated from cycling animals in vitro and this study was conducted using pig follicles (Grochowalski *et al.*, 2000). The follicle culture system we used in this study has been well characterized and has been proven to be an excellent model for in vitro testing of ovarian function in reproductive toxicology studies (Cortvrindt and Smitz, 2002; Gupta *et al.*, 2006). The cultured follicles maintain much of their steroidogenic and proliferative capacity throughout the 96 hour culture period; maintaining the presence of granulosa cells, theca cells, a basement membrane and oocytes in meiotic arrest (Cortvrindt *et al.*, 1997). Thus, unlike studies utilizing primary granulosa cells or human luteinized granulosa cells in culture, all the functional compartments of the follicle are present in our system. In addition, we also chose a series of TCDD doses previously shown to have effects on ovarian tissues and cells in culture, which also encompass environmentally relevant (0.1 and 1nM) as well as pharmacological exposures (10nM and 100nM), so that we could determine a dose response for TCDD in our culture system (Birnbaum and DeVito, 1995; Gregoraszczuk, 2002).

Proliferation of follicular cells contributes to the overall growth of follicles (Hirshfield, 1991). Our data indicate that TCDD does not affect growth of follicles, suggesting that it does not affect proliferation of granulosa cells in mice. These data differ from those obtained using pig follicles and from in vivo dosing experiments in rats. Specifically, Grochowalski et al. found that TCDD reduced the percentage of proliferating cells in porcine follicles over

time and that this was attributed to increasing accumulation of TCDD in the tissue over time (Grochowalski *et al.*, 2000). Heimler *et al.* found a decrease in the number of antral follicles without an increase in atresia in the ovaries of TCDD exposed rats suggesting an anti-proliferative effect (Heimler *et al.*, 1998b). While it is unclear why our results differ from others, it is likely due to species differences or differing experimental conditions.

We also found that TCDD alters steroidogenesis in mouse follicles in a non-monotonic/ U-shaped dose response manner. These data are consistent with studies in other species and culture systems (Dasmahapatra *et al.*, 2000; Gregoraszczuk, 2002; Grochowalski *et al.*, 2000; Heimler *et al.*, 1998a). Specifically, in studies utilizing isolated porcine thecal and granulosa cell co-cultures, TCDD exposures of 0.1nM or 10nM resulted in reduced E2 accumulated in the media, while only the 10nM exposure resulted in a significant reduction in progesterone in the media after 96 hour cultures (Grochowalski *et al.*, 2001). Further, unlike our results for E2 levels in the media after TCDD exposure, Gregoraszczuk *et al.* observed a dose response decrease in E2 levels with increasing TCDD concentration up to 100nM in the media after only 48 hours of culture (Gregoraszczuk, 2002). We did not observe a significant decrease after exposure to the highest dose of TCDD (100nM) after 96 hours. This difference could be due to time dependent effects of TCDD on steroidogenesis in follicles. Studies comparing single versus prolonged exposures to TCDD on steroidogenesis in pig follicles have revealed a varying response with a decrease and an increase in testosterone levels, respectively (Grochowalski *et al.*, 2000). It is also possible that at pharmacological doses of TCDD, steroidogenesis is able to recover after prolonged TCDD exposure such as was demonstrated in porcine luteal cells and in human luteinized granulosa cells in culture (Gregoraszczuk *et al.*, 2000; Heimler *et al.*, 1998a). This could be an explanation for the lack of effect we observed on the levels of P4, A4, T and E2 assayed from the mouse follicles exposed to the highest pharmacological dose of TCDD (100nM) in the media after 96 hours in culture. Interestingly, only the 1nM dose of TCDD significantly inhibited the accumulation of P4 in the media after 96 hours culture. These results suggest that like hormones and other environmental contaminants that act as endocrine disruptors, TCDD may have multiple modes of action in mouse antral follicles depending on the dose (Gierthy, 2002; Gore *et al.*, 2006).

Since P4 accumulation in the media was decreased after TCDD exposure and P4 synthesis is early in the steroidogenic pathway, we investigated whether we could by-pass this effect with pregnenolone substrate co-treatment with the 1nM dose of TCDD. Addition of pregnenolone substrate restored P4 in the media to control levels, suggesting that TCDD could be acting on StAR or P450scc function in mouse antral follicles. Similar to our findings, Gregoraszczuk *et al.* were able to restore P4 levels after co-treatment with pregnenolone and TCDD in porcine granulosa thecal cell co-cultures (Gregoraszczuk, 2002). Supportive of this observation, TCDD has been shown to activate the AHR, leading to transcriptional regulation of the promoter activities of P450scc, the mitochondrial enzyme responsible for cleaving cholesterol to pregnenolone in the steroidogenic pathway (Dasmahapatra *et al.*, 2000). Specifically, Dasmahapatra *et al.* found a reduction in the level of P450scc mRNAs in isolated rat granulosa cells exposed to TCDD (Dasmahapatra *et al.*, 2000). Contrary to this finding, studies investigating the effects of TCDD on steroidogenesis in rat testes and adrenal glands in vivo suggest that TCDD may not directly act on inhibiting P450scc activity or levels, but may inhibit the mobilization of cholesterol to P450scc; ultimately leading to a decrease in hormone levels (DiBartolomeis *et al.*, 1987; Kleeman *et al.*, 1990; Moore *et al.*, 1991). Though it was not directly tested in this study, it is likely that at the 1nM dose, TCDD does not affect downstream enzymes in the steroidogenic pathway since pregnenolone co-treatment also restored A4, T, and E2 accumulation in the media.

Since androgen and E2 levels were also decreased at doses of TCDD when P4 levels were not affected, it is likely that TCDD acts downstream of pregnenolone formation in the steroidogenic pathway in the follicle as well. This is supported by several studies utilizing porcine granulosa thecal cell co-cultures, rat granulosa cell cultures and human luteinized granulosa cell cultures. Gregoraszczuk found evidence of reduced aromatase activity in porcine granulosa and thecal cell co-cultures after TCDD exposure (Gregoraszczuk, 2002). Similarly, studies conducted using FSH-stimulated rat granulosa cells found that the addition of A4 partially restored E2 levels. Upon further investigation, they found that TCDD inhibited the FSH-stimulated increase in aromatase activity usually exhibited by these cells, indicating possible actions on this enzyme as well as the follicle stimulating hormone receptor (Dasmahapatra *et al.*, 2000). Studies exposing human luteinized granulosa cells (hLGCs) to TCDD in culture also found a decrease in E2 accumulation in the media after TCDD exposure, but contrary to the studies using rat granulosa cells, Morán *et al.* did not find a decrease in aromatase levels or activity (Morán *et al.*, 2000). Instead, they demonstrated that the reduced E2 was the result of an impairment in the supply of aromatizable androgens due to a decrease in the lyase activity of 17 α hydroxylase/ 17,20 lyase cytochrome P450 (CYP17A1) enzyme as well as decreased levels of CYP17A1 protein levels in hLGCs (Morán *et al.*, 2003a; Morán *et al.*, 2003b).

Other mechanisms by which TCDD could be affecting hormone levels in the follicle are through an AHR pathway that drives transcriptional activation of genes responsible for E2 catabolism. AHR transcriptional activation via TCDD ligand binding has been shown to increase catabolism of E2 by inducing the cytochrome P450 monooxygenases such as CYP1B1 (Badawi *et al.*, 2001; Hayes *et al.*, 1996). CYP1B1 is responsible for converting E2 to the catecholestrogen 4-hydroxyestradiol (Badawi *et al.*, 2001). CYP1B1 is expressed in the ovary in the absence of exogenous ligands and it is hormonally regulated and inducible by TCDD (Bhattacharyya *et al.*, 1995; Brake and Jefcoate, 1995; Dasmahapatra *et al.*, 2002; Otto *et al.*, 1992; Savas *et al.*, 1994; Shen *et al.*, 1993; Shen *et al.*, 1994; Sutter *et al.*, 1994). Additionally, it has been shown in porcine granulosa cells that the AHR shuttles from the cytoplasm into the nucleus in the presence of TCDD, evidence of transcriptional activity in the ovary (Wojtowicz *et al.*, 2005). Thus, we tested the hypothesis that TCDD exposure transcriptionally activates the AHR and induces *Cyp1b1* leading to increased levels of *Ahr* and *Cyp1b1* mRNA in our cultured antral follicles. Interestingly, we did not observe an increase in *Ahr* transcript levels after TCDD exposure similar to what was observed in rat and porcine granulosa cells in culture (Dasmahapatra *et al.*, 2001; Wojtowicz *et al.*, 2005). This could be due to the longer exposure we employed, or because the presence of thecal cells may conceal the effects on granulosa cells in antral follicle cultures. Currently, very little is known about how TCDD regulates AHR levels in the ovary, though it has been suggested that the aryl hydrocarbon receptor repressor (AHRR) plays a role in the absence of exogenous ligands, and this regulation might be at the protein level since *Ahr* mRNA levels remain unchanged in ovaries from gonadotropin primed mice (Baba *et al.*, 2005).

Although we did not find any significant changes in *Ahr* transcript levels, we did observe a 3-4 fold increase in *Cyp1b1* levels following 96 hours of TCDD exposure (0.1-100nM), which is slightly higher than was observed in isolated rat granulosa cells exposed to TCDD for only 6 hours (3.1nM) (Dasmahapatra *et al.*, 2002). These findings strongly suggest and agree with the majority of published data that in addition to the effects of TCDD on the steroidogenic pathway, TCDD exposure may also lead to increased catabolism of E2 to catecholestrogens in the ovary.

This idea is further supported by studies that found that acute exposure to TCDD in the ovariectomized rat decreases the elimination constant and clearance of E2 (Petroff and Mizinga, 2003). This could be one explanation for why contrary to our findings in vitro,

circulating E2 levels were unaltered following in vivo TCDD exposure in CD-1 mice (DeVito et al., 1992). It could be that TCDD exposure decreases E2 synthesis and increases E2 catabolism in the mouse ovary, while it does not change circulating levels due to a slower clearance rate. The lack of effect on E2 levels after the in vivo TCDD exposure in CD-1 mice could also be related to the route of administration, the age of the animals and the stage of the reproductive cycle. Studies conducted using rats show that circulating E2 levels do not change in a gonadotropin primed immature hypophysectomized model with a very high single exposure to TCDD, while chronic exposure to low doses in utero lead to lower levels of circulating E2 in pubertal life (Son et al., 1999; Franczak et al., 2006). Though TCDD metabolites have not been measured from follicles exposed to TCDD in vitro, ovaries express a battery of enzymes known to metabolize TCDD such as CYP1B1 (Bengtsson AND Rydstrom; 1983; Dasmahapatra et al. 2002). Thus, it is likely that some of the local effects of TCDD on the ovary could be due to actions of its metabolites on steroidogenesis as well.

In conclusion, we have demonstrated that TCDD disrupts sex steroid hormone production in a non-monotonic dose response manner without altering growth of mouse antral follicles. We were able to by-pass the disrupted steroid hormone production with pregnenolone co-treatment, suggesting that TCDD may be involved in inhibiting the mobilization of cholesterol to the inner mitochondrial membrane and/or in the conversion of cholesterol to pregnenolone in antral follicles. These actions of TCDD on steroid hormone metabolism are likely through an AHR pathway, as demonstrated by induction of the *Cyp1b1* transcript and constitutive expression of the *Ahr* in our culture system. Overall, the experimental results reported here and by others demonstrate complex actions of TCDD toxicity in the ovary. Since humans and animals are exposed to a mixture of dioxins and other environmental toxicants, it will be important in the future to study the toxicity of these environmentally relevant mixtures.

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Highlights

- TCDD disrupts sex steroid hormone levels, but not growth of antral follicles.
- Pregnenolone co-treatment by-passes TCDD-induced steroid hormone disruption.
- TCDD affects steroid hormone levels through an AHR pathway in antral follicles.

Fig 1. Effect of TCDD on mouse antral follicle growth in vitro

Antral follicles were mechanically isolated from young cycling CD1 mice and exposed for 96 hours in culture to TCDD (0.1nM-100nM) or vehicle alone. The follicle diameters were measured every 24 hours for a period of 96 hours. Follicle growth was plotted as percent change in diameter over time from the beginning of the culture. The graph represents the means $\pm$ SEM from at least three individual experiments (n=8-16 follicles per treatment group per individual experiment).

Fig 2. Effect of TCDD on the gross morphology of mouse antral follicles in vitro

Antral follicles were mechanically isolated from young cycling CD1 mice and exposed for 96 hours to TCDD (0.1nM-100nM) or vehicle alone. Digital images were taken of mouse antral follicles at 24 hours and at 96 hours of culture using a digital camera (ProgRes CT3, Jenoptik, Germany) attached to an inverted microscope using the 10X objective (Olympus CK40). NT= non-treated.

Fig 3. Effect of TCDD on mouse antral follicle sex steroid hormone secretion in vitro
Antral follicles were mechanically isolated from young cycling CD1 mice and exposed for 96 hours in culture to TCDD (0.1nM-100nM) or vehicle alone. After the 96 hour culture period, media were collected and subjected to hormone assays for (A) progesterone, (B) androstenedione, (C) testosterone, and (D) estradiol levels. The graphs represent the mean hormone levels $\pm$ SEM from 8-23 culture wells from a minimum of three separate follicle culture experiments. Bars with different letters are significantly different from each other ($p < 0.05$).

Fig 4. Effect of pregnenolone substrate co-treatment with TCDD on mouse antral follicle steroid hormone secretion in vitro

Mouse antral follicles were treated with vehicle (DMSO), pregnenolone (10 μ M), TCDD (1nM), or TCDD (1nM) and pregnenolone (10 μ M) for 96 hours in culture. After the 96 hour culture, media were collected and subjected to hormone assays for (A) progesterone, (B) androstenedione, (C) testosterone, and (D) estradiol levels. The graphs represent the mean hormone levels $\pm$ SEM from 8-23 culture wells from a minimum of three separate follicle culture experiments. Bars with different letters are significantly different from each other ($p < 0.05$). Preg = pregnenolone.

Fig 5. Effect of pregnenolone substrate co-treatment with TCDD on mouse antral follicle growth in vitro

Mouse antral follicles were treated with vehicle (DMSO), pregnenolone (10 μ M), TCDD (1nM), or TCDD (1nM) and pregnenolone (10 μ M) for 96 hours in culture. The follicle diameters were measured every 24 hours for a period of 96 hours. Follicle growth was plotted as percent change in diameter over time from the beginning of the culture. The graph represents the means $\pm$ SEM from at least three individual experiments (n=8-16 follicles per treatment group per individual experiment).

Fig 6. Effect of TCDD exposure on the expression of *Cyp1b1* and *Ahr* transcripts in antral follicles in vitro

At the end of each of the 96 hour cultures, 8-16 follicles from each treatment group were pooled and immediately snap frozen in liquid nitrogen and assayed by real time qPCR for levels of (A) *Cyp1b1* and (B) *Ahr*. Levels of *Cyp1b1* and *Ahr* were normalized to beta actin (*Actb*). Data are expressed as mean relative expression ratios $\pm$ SEM calculated from 3 - 4 separate culture experiments. Bars with different letters are significantly different from each other ($p < 0.05$).